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AUTHORITY
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Award Number: DAMD17-98-1-8232

TITLE: Development of Strategies to Manipulate ErbB Receptor
Heterodimerization from a Quantitative Analysis of
Receptor/Ligand Relationships

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REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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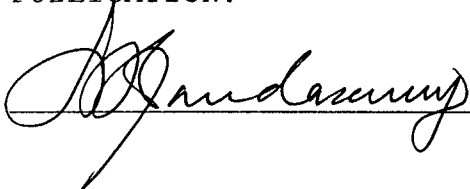
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 99 - 30 Jun 00)	
4. TITLE AND SUBTITLE Development of Strategies to Manipulate ErbB Receptor Heterodimerization from a Quantitative Analysis of Receptor/Ligand Relationships			5. FUNDING NUMBERS DAMD17-98-1-8232	
6. AUTHOR(S) Mark Lemmon, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Philadelphia, Pennsylvania 19104-3246 E-MAIL: mlemmon@mail.med.upenn.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Jul 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Members of the erbB family of receptor tyrosine kinases include the epidermal growth factor (EGF) receptor and erbB2 (also known as HER-2/Neu) that is found overexpressed in many human breast cancer cases. The aim of our studies is to understand the mechanism by which growth factors activate these receptors. If this mechanism can be understood in detail, it should be possible to design approaches to block inappropriate receptor activation, which occurs in many breast cancers. While EGF activates erbB1 (the EGF receptor) by directly inducing its homodimerization, the same growth factor activates erbB2 by inducing the formation of hetero-oligomeric complexes between erbB1 and erbB2. We have shown that the isolated extracellular domain of erbB1 is sufficient for EGF-induced homodimerization of that receptor. By contrast, isolated extracellular domains are not sufficient to recapitulate the hetero-oligomerization of erbB receptors that has been observed <i>in vivo</i> . These findings argue that erbB receptor homo- and hetero-oligomerization occur through different mechanisms. Using a variety of approaches for directly visualizing erbB receptor homo- and hetero-oligomerization in living cells, we are now investigating the origin of this mechanistic difference, and defining the regions of the receptors that participate. Answers to these questions will likely suggest new avenues for pharmacological intervention when erbB receptors are inappropriately activated.				
14. SUBJECT TERMS Breast Cancer; erbB receptor; heterodimerization; ligand binding; growth factor; neuregulin				15. NUMBER OF PAGES 27
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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INTRODUCTION

Cellular responses to many extracellular factors that control cell growth and differentiation are mediated by cell-surface receptors with tyrosine kinase activity. The mechanism of transmembrane signaling by receptor tyrosine kinases involves an initial ligand-induced receptor dimerization or oligomerization event that leads to activation of the intracellular tyrosine kinase domain. For a given class of receptor tyrosine kinases, ligand-induced oligomerization can involve two receptors that are the same (homo-oligomerization) or different (hetero-oligomerization). It is now appreciated that hetero-oligomerization provides a mechanism for increasing the diversity of signaling through a given family of receptors. In our studies we are focusing on the four known receptors of the epidermal growth factor (EGF) receptor family - known as erbB1, erbB2, erbB3, and erbB4. The erbB receptors have been implicated in many human cancers. In particular, erbB2 (also known as Neu, or HER-2) is strongly implicated in breast cancer. ErbB2 is found over-expressed in around 30% of human breast cancer cases, and is the direct target of Herceptin, which is now an important component of our armory in fighting many breast cancers. Aberrant overexpression of any member of the erbB receptor family can disrupt normal growth signaling, in many cases leading to uncontrolled cell proliferation, and thus cancers. There are at least 12 different ligands that signal through the erbB receptor tyrosine kinases, and these include EGF, TGF α , and the neuregulins. The erbB ligands differ in their receptor-binding characteristics, and appear to induce the formation of distinct combinations of erbB receptor homo- and hetero-oligomers on the cell surface. The specific biological activities of the ligands are thought to arise from these differences. We are trying to understand precisely how the multiple different ligands induce formation of particular receptor dimers. For the EGF receptor (erbB1), we showed that the extracellular domain is sufficient for ligand-induced dimerization, and that two EGF molecules are required to form the dimer. Through biophysical analyses of the other erbB receptor extracellular domains, produced in a baculovirus expression system, we have compared ligand-induced receptor homo- and heterodimerization by EGF and neuregulin-1 β (NRG1- β), the results of which will be published in the *EMBO Journal* in September 2000. Our findings show that 'transmodulation' of erbB receptors through hetero-oligomerization must occur through a mechanism that is different from the accepted ligand-induced homodimerization model established for activation of the EGFR (erbB1) by EGF. Furthermore, while ligand-induced heterodimerization may be relevant for erbB2/erbB3/erbB4, it appears not to be important for the EGF receptor (erbB1). Having established this mechanistic difference *in vitro*, we are now studying oligomerization of the intact receptors *in vivo*, to understand just how erbB receptor transmodulation does occur. By developing this understanding, we hope that approaches will be suggested for specifically modulating erbB receptor signaling when it is disrupted in human cancers.

BODY OF PROGRESS REPORT

Progress for each task is detailed following description of the task

Task 1.

To determine quantitatively the hierarchy of erbB receptor homo- and heterodimers induced by each ligand in the EGF and NRG family

All proposed aspects of this task were completed in the first year of the award, as described in the July 1999 progress report. The results of this analysis are to be published in the *EMBO Journal* in September 2000 (Ferguson *et al.*, 2000). A copy of the proofs of this paper is attached as Appendix 1.

Whereas the erbB1 and erbB4 extracellular domains quantitatively form *homodimers* upon binding to their respective ligands, our *in vitro* biophysical studies showed that isolated erbB receptor extracellular domains do not *heterodimerize* upon ligand binding. As discussed in detail by Ferguson *et al.* (2000 - see also Appendix 1), we therefore conclude that hetero-oligomers of full-length erbB receptors reported in the literature, which are responsible for erbB receptor 'transmodulation', do NOT resemble the homodimers of erbB1 and erbB4 induced by EGF and NRG1- β 1.

This finding alters the details (but not basic approaches or scope) of the question that was originally asked in Task 2 of the Statement of Work.

Task 2.

To test the prediction of studies using s-erbB proteins in a cellular context

Our original approach was:

- from the studies of s-erbB heterodimerization, we will predict which combinations of erbB receptors will create high-affinity binding sites for which ligands. These predictions will be tested by studying ligand binding to breast cancer cell-lines that express known combinations of erbB receptors (months 18-24).

Since our *in vitro* studies show the simple model of extracellular domain-mediated erbB receptor heterodimerization to be wrong, a different set of precise questions must first be answered:

1. Can hetero-oligomerization of intact erbB receptors be visualized directly in living cells ?
2. Are hetero-oligomers dimeric, or of higher order (*e.g.* heterotetramers) ?
3. Which domains in the erbB receptors are responsible for their hetero-oligomerization ?

For example, one model for EGF-induced activation of erbB2 (Huang *et al.*, 1998) suggests that an EGF-induced homodimer of the EGF receptor (erbB1) provides a 'dimerization

surface' for erbB2 - thus causing homodimerization and activation of erbB2 'by proxy' (in the context of a heterotetramer). We will test this hypothesis as part of our studies.

To address these questions, we have modified our direction for studying erbB receptor heterodimerization *in vivo* with the following tasks:

- develop approaches for studying the hetero-oligomerization of intact erbB receptors in live cells (months 12-36)
- investigate the role of portions of erbB receptors other than their extracellular domains on both homo- and hetero-dimerization (months 12-36)

We have made progress in both of these new directions, as outlined below:

A develop approaches for studying the hetero-oligomerization of intact erbB receptors in live cells (months 12-36)

We have chosen to pursue two different approaches to achieving this difficult, but very important, goal. In one, we have employed 'protein complementation' approaches. In the other, we are developing ways of visualizing hetero-oligomerization using green fluorescent protein (GFP) derivatives.

Helen Blau's laboratory recently published an interesting approach for monitoring EGF receptor homodimerization in living NIH 3T3 cells (Blakely *et al.*, 2000). Advantage is taken of two different mutants of *E. coli* β -galactosidase ($\Delta\alpha$ and $\Delta\omega$), neither of which is active in isolation. The two mutants can combine to form an enzymatically active β -galactosidase, but only if they are forced to associate with one another - *e.g.* by fusion to a dimerizing species. Blau's group used this approach to study homodimerization of the EGF receptor. They replaced the intracellular domain of the EGF receptor with the $\Delta\alpha$ or $\Delta\omega$ mutant of β -galactosidase, and co-expressed EGFR- $\Delta\alpha$ and EGFR- $\Delta\omega$ in NIH 3T3 cells. EGF-dependent β -galactosidase activity could be seen in the resulting cells, but only when the EGFR chimeras were expressed at very low levels.

We attempted to adapt this approach for studying erbB receptor hetero-oligomerization in living cells. Having generated constructs, and cell-lines expressing the chimeras, we quickly realized that the approach is not very robust. Even at conservative levels of expression, the background of EGF-independent β -galactosidase activity in cells expressing both EGFR- $\Delta\alpha$ and EGFR- $\Delta\omega$ was very high. We were able to see EGF-dependent increases in β -galactosidase activity, but not more than 10% over background when EGF receptor chimeras were expressed at approximately 'normal' levels. We therefore chose not to pursue this approach for analysis of hetero-oligomerization, since it was clear that expression levels would have to be controlled to an extent that would make experiments impossible to interpret. We believe that simply anchoring the

$\Delta\alpha$ and $\Delta\omega$ mutants of β -galactosidase to the plasma membrane increases their respective local concentrations to an extent that promotes their spontaneous interaction and complementation.

A graduate student in the laboratory is now working instead on a more promising approach that employs the interleukin-2 (IL-2) receptor. Nelson *et al.* (1994) showed in the IL-2-dependent T-cell line CTLL2, that heterodimerization of the cytoplasmic domains of IL-2R β and IL-2R γ is necessary and sufficient for IL-2 receptor (IL-2R) signaling in T cells. This was shown with chimeric receptors containing the extracellular domain of the stem cell factor (SCF) receptor (c-kit), and the transmembrane and cytoplasmic domains of IL-2R β (kit/2 β) or IL-2R γ (kit/2 γ). SCF causes homodimerization of the c-kit extracellular domain (similar to EGF-induced dimerization of s-erbB1). In CTLL2 cells expressing both kit/2 β and kit/2 γ , SCF induces heterodimerization of the two chimera, and induces an IL-2-like response. By contrast, in cells expressing only one of the two chimeras, there was no proliferative response to SCF. We have recently generated equivalent erbB1/2 β and erbB1/2 γ chimeras with which we are about to perform similar experiments. Once this system is established, we will use it to determine whether erbB1 and erbB2 can form heterodimers - studying CTLL2 cells expressing erbB2/2 β and erbB1/2 γ . In addition, using erbB2/2 β and erbB2/2 γ chimeras, and co-expressing these with full-length erbB1, we will determine whether EGF can induce homodimerization of erbB2 'by proxy', as suggested by Huang *et al.* (1998).

Finally, we have begun approaches to monitoring erbB receptor heterodimerization in live cells using fluorescence resonance energy transfer between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Siegel *et al.* (2000) have recently used FACS analysis and confocal microscopy to investigate oligomerization of Fas using chimeras containing CFP and YFP respectively. Following this example, we have generated constructs for expression of erbB1/CFP and erbB1/YFP chimeras, and have begun developing approaches to analyzing fluorescence energy transfer.

With reagents and constructs now in place, we anticipate being able to investigate erbB receptor oligomerization directly, in living cells, over the coming year. This advance will allow us to address many questions that are inaccessible in studies of extracellular domains alone, and will permit a detailed study of hetero-oligomerization specificity and determinants of receptor-receptor interactions.

B. investigate the role of portions of the erbB receptors other than their extracellular domains on both homo- and hetero-dimerization (months 12-36)

Although ligand-induced erbB1 and erbB4 homodimerization can be recapitulated with the isolated extracellular domain of the receptors, erbB3 homodimerization cannot, and neither can heteromeric interactions. This result raises questions concerning the roles of other domains in receptor-receptor interactions. A systematic study of individual domain contributions will be

possible, through domain-swapping experiments, once a system for direct analysis of erbB receptor oligomerization in living cells has been developed (A above). However, there are several reasons to suspect that the single transmembrane domain of each erbB receptor may participate in homo- and/or hetero-oligomerization of the receptor, especially with erbB2/neu. Indeed, specific interactions between transmembrane (TM) α -helices are well known to be both highly specific and strong in certain cases (Lemmon and Engelman, 1994). Furthermore, mutations in the TM domain of the rat erbB2 homolog (Neu) are known to be capable of inducing its constitutive activation (Hynes and Stern, 1994), and studies of erbB3 and erbB2 hetero-oligomerization have implicated TM domains in receptor-receptor interactions (Tzahar *et al.*, 1997).

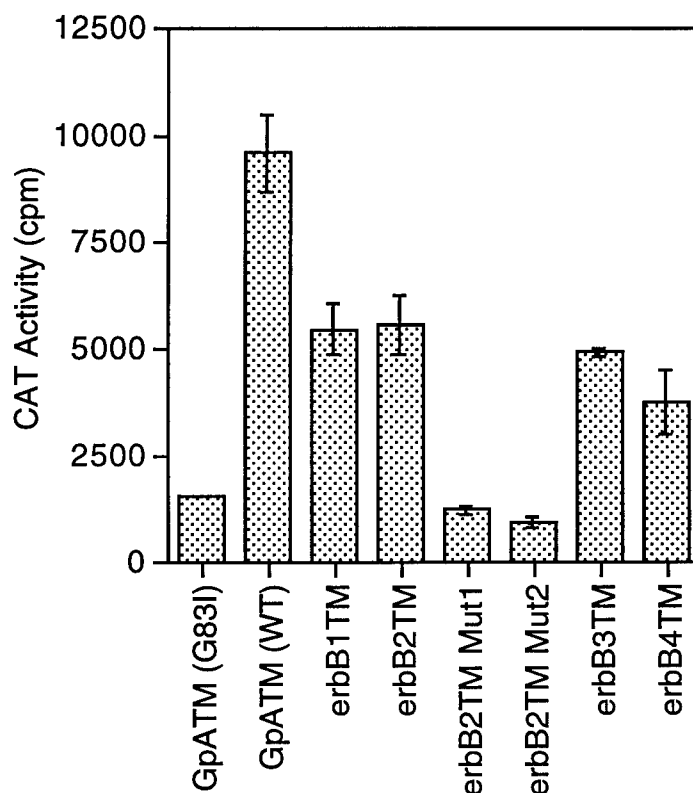


Figure 1

CAT activity in *E. coli* lysates expressing ToxR chimeras of different GpA and erbB receptor TM domains. The G83I mutation in the GpA TM domain abolishes GpA TM dimerization (Lemmon *et al.*, 1992). Error bars are standard deviations. See text for details of erbB2TM mutants.

We have begun investigations of homo- and hetero-oligomerization of the erbB receptor TM domains, using a newly developed approach that monitors their ability to associate in the plasma membranes of bacteria (Russ and Engelman, 1999). In this system, named 'TOXCAT', the transmembrane domains are fused to the N-terminal DNA binding domain of the ToxR transcription factor, which can act as a transcription factor when membrane anchored, and requires dimerization in order to induce transcription from the *ctx* promoter. TM domain/ToxR chimeras are expressed in *E. coli* containing a chloramphenicol acetyltransferase (CAT) gene under control

of the *ctx* promoter. If the TM domain oligomerizes, the CAT gene is transcribed, and CAT activity can be assayed using standard procedures. Russ and Engelman (1999) developed this system using the TM domain of human glycophorin A (GpA), which forms very stable dimers (Lemmon *et al.*, 1992), as a model system.

Using this system, we have found that the TM domains of erbB1, erbB2, erbB3, and erbB4 all form homodimers (Figure 1). Results are normalized for expression levels, and correct insertion of the chimeras into the plasma membrane was demonstrated as described by Russ and Engelman (1999).

The transmembrane domains tested are as follows:

GpA	I T L I I F <u>G</u> V M A <u>G</u> V I G T I L L I S Y G I
GpA (G83I)	I T L I I F <u>G</u> V M A I V I G T I L L I S Y G I
erbB1	I A T G M V <u>G</u> A L L L L V V <u>A</u> L G I <u>G</u> L F M
erbB2	V V <u>S</u> A V V <u>G</u> I L L V V V L <u>G</u> V V F <u>G</u> I L I
erbB2Mut1	V V <u>S</u> A V V V I L L V V V L <u>G</u> V V F <u>G</u> I L I
erbB2Mut2	V V <u>S</u> A V V <u>G</u> I L L V V V L <u>G</u> V V F V I L I
erbB3	L T M A L <u>T</u> V I A <u>G</u> L V V I F M M L G G T F L
erbB4	L I A A <u>G</u> V I G <u>G</u> L F I L V I V <u>G</u> L T F <u>A</u> V Y

In the case of GpATM, a motif GxxxGxxxT was found to be critical for TM helix dimerization. In particular, it has been shown that the GxxxG (G's underlined) motif is central in the helix-helix interface. Mutation of one of the glycines in this motif (G83 if GpA) to isoleucine abolishes dimerization. As shown above (underlined residues), erbB1TM, erbB2TM, and erbB4TM all have two similar motifs (small-x-x-x-small), while erbB3TM has one. We have mutated one glycine in each of the two motifs seen in erbB2TM to valine, to generate erbB2Mut1 and erbB2Mut2 respectively. As shown in Figure 1, either of these mutations abolishes erbB2TM dimerization.

We have introduced these mutations into full-length erbB2, and are now testing their effects on erbB2 transmodulation by erbB1, and on the ability of erbB2 overexpression to transform cells. These experiments will be completed in the next 12 months.

Finally, we have begun to adapt the TOXCAT approach to permit analysis of heteromeric TM domain interactions.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that erbB receptor extracellular domains are sufficient for ligand-induced homodimerization of the receptors, but do not recapitulate erbB receptor *hetero*-oligomerization.
- Established reagents for two different approaches for analyzing ligand-induced hetero-oligomerization of intact erbB receptors in living cells.
- Demonstrated that the transmembrane domains of erbB receptors are capable of homodimerization that (at least in the case of erbB2TM) can be specifically disrupted with single amino acid substitutions.
- Generated mutants of intact erbB2 for analyzing the role of TM domain homo-oligomerization in erbB2 transmodulation and activation by simple over-expression.

REPORTABLE OUTCOMES

1. Results were presented as a poster at the Era of Hope meeting in Atlanta GA, June 8-11, 2000
2. Manuscript entitled: "Extracellular domains drive homo- but not hetero-dimerization of erbB receptors, by K.M. Ferguson, P.J. Darling, M. Mohan, T.L. Macatee, and M.A. Lemmon, was accepted for publication in the *EMBO Journal*, and will be published in No 17 of the current volume (19) in September 2000. Page proofs of the article are provided as Appendix 1.
3. Awarded R21 grant (R21-CA87182) by the National Cancer Institute, for "Structural Studies of ErbB/Her Receptor Dimerization" Lemmon P.I.

CONCLUSIONS

Our studies to date alter the view of erbB receptor activation by EGF and NRG family members. It has been argued that the four erbB receptors can form 10 different dimers (counting homo- and hetero-dimers), which differ in their signaling potential. Different erbB ligands have been proposed to select different combinations of these 10 dimers, and this selection is suggested to define the signaling specificity of each particular ligand.

Our findings show that this view is incorrect. While homodimers of erbB1 and erbB4 are driven by simple (likely bivalent) binding of ligands to receptor extracellular domains, heteromeric receptor interactions are more complex - requiring either the formation of higher-order oligomers or the participation of other portions of erbB receptors. We are now in the process of determining the origins of these differences using a variety of approaches in living cells.

In the past year, we have completed and published our studies of ligand-induced dimerization of erbB extracellular domains, and we have generated the tools and preliminary data for a systematic study of erbB receptor oligomerization in a cellular context.

So What ?

A key aim in breast cancer is to inactivate or otherwise remove erbB2/Neu/Her2 in the 30% or so of cases where its over-expression is seen. This is the basis of the efficacy of Herceptin. Our studies are altering and refining the current understanding of how erbB2/Her2 is regulated in cells. In particular, it now appears that erbB2 is transmodulated by EGF receptor through a mechanism that is quite different from activation of the EGF receptor itself. Understanding this mechanism, and how it differs from activation of the EGF receptor, will allow us to consider approaches for specifically reversing erbB2 activation (leaving EGF receptor activation etc unaffected) when it occurs inappropriately in breast cancers. The more sophisticated and detailed our understanding of the mechanism (and its structural basis), the more specific and refined will be the pharmacologic agents one can design to intervene in the process.

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Extracellular domains drive homo- but not hetero-dimerization of erbB receptors

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Many different growth factor ligands, including epidermal growth factor (EGF) and the neuregulins (NRGs), regulate members of the erbB/HER family of receptor tyrosine kinases. These growth factors induce erbB receptor oligomerization, and their biological specificity is thought to be defined by the combination of homo- and hetero-oligomers that they stabilize upon binding. One model proposed for ligand-induced erbB receptor hetero-oligomerization involves simple heterodimerization; another suggests that higher order hetero-oligomers are 'nucleated' by ligand-induced homodimers. To distinguish between these possibilities, we compared the abilities of EGF and NRG1- β 1 to induce homo- and hetero-oligomerization of purified erbB receptor extracellular domains. EGF and NRG1- β 1 induced efficient homo-oligomerization of the erbB1 and erbB4 extracellular domains, respectively. In contrast, ligand-induced erbB receptor extracellular domain hetero-oligomers did not form (except for s-erbB2-s-erbB4 hetero-oligomers). Our findings argue that erbB receptor extracellular domains do not recapitulate most heteromeric interactions of the erbB receptors, yet reproduce their ligand-induced homo-oligomerization properties very well. This suggests that mechanisms for homo- and hetero-oligomerization of erbB receptors are different, and contradicts the simple heterodimerization hypothesis prevailing in the literature.

Keywords: dimerization/growth factor/scattering/signaling/tyrosine kinase

Introduction

The epidermal growth factor (EGF) receptor is the prototype of the erbB family of receptor tyrosine kinases (RTKs) that also includes erbB2 (HER-2 or Neu), erbB3 (HER-3) and erbB4 (HER-4) (Carraway and Cantley, 1994; Alroy and Yarden, 1997; Riese and Stern, 1998). Each erbB receptor contains an extracellular ligand-binding domain of 600-630 amino acids, a single transmembrane α -helix, plus an intracellular domain of ~600 amino acids that includes the tyrosine kinase and regulatory sequences (Schlessinger and Ullrich, 1992). It was established more than a decade ago for the EGF receptor (erbB1) that growth factor-induced receptor

oligomerization is critical for transmembrane signaling (Schechter *et al.*, 1979; Schlessinger, 1979; Yarden and Schlessinger, 1987a,b). It is now generally accepted that the cytoplasmic tyrosine kinases of two (or more) RTKs in a growth factor-induced dimer (or larger oligomer) mutually activate one another through transphosphorylation (Honegger *et al.*, 1990; Lemmon and Schlessinger, 1994; Heldin, 1995; Hubbard *et al.*, 1998). Several downstream signaling molecules are then recruited to the phosphorylated receptor, specified by its complement of regulatory tyrosine phosphorylation sites (Songyang *et al.*, 1993; Schlessinger, 1994).

Many cells co-express multiple members of the erbB receptor family, which can form both homo- and hetero-oligomers upon stimulation with growth factor ligands (Heldin, 1995). Oligomers containing almost every possible pairwise combination of erbB receptors have now been reported (reviewed by Carraway and Cantley, 1994; Alroy and Yarden, 1997; Riese and Stern, 1998). The earliest evidence for hetero-oligomerization of erbB receptors came from the finding that erbB2 can be activated by EGF, despite the fact that it does not bind directly to this ligand. EGF is only able to activate erbB2 when erbB1 is also present in the same cell, suggesting 'transmodulation' of erbB2 as a result of its EGF-induced hetero-oligomerization with erbB1 (King *et al.*, 1988; Stern and Kamps, 1988; Wada *et al.*, 1990; Goldman *et al.*, 1990; Spivak-Kroizman *et al.*, 1992).

There are >10 distinct ligands that activate erbB receptors. Three of these have been classified as 'EGF agonists' (Riese and Stern, 1998), since they bind directly to only erbB1 [EGF, transforming growth factor- α (TGF- α) and amphiregulin]. Four (or more) of the ligands are specific for erbB3 and/or erbB4 (the neuregulins; NRGs), while a further three have been classified as 'bispecific' and bind directly to both erbB1 and erbB4 [betacellulin, epiregulin and possibly heparin-binding EGF-like factor (HB-EGF)] (Riese and Stern, 1998; Harari *et al.*, 1999; J.T.Jones *et al.*, 1999, and references therein). The EGF agonists activate erbB1 when it is expressed alone, but also transmodulate erbB2, erbB3 and erbB4 in an erbB1-dependent manner. Similarly, the NRGs activate erbB4 directly, but can also transactivate erbB1 or erbB2 when erbB4 or erbB3 are also present (Riese *et al.*, 1995). Finally, the bispecific ligands appear to activate erbB1 and erbB4 when either is expressed alone, and to transmodulate erbB2 and erbB3 via these receptors (reviewed by Alroy and Yarden, 1997; Riese and Stern, 1998). ErbB2, which is of particular medical interest as a target of breast cancer therapies (Sliwkowski *et al.*, 1999), has no known ligand and can only be activated *in trans* by ligands in these three classes. In fact, erbB2 is considered to be a preferred hetero-oligomerization partner for all of the

other erbB receptors (Karunakaran *et al.*, 1996; Graus-Porta *et al.*, 1997).

Several possible mechanisms for erbB receptor transmodulation have been considered. In the simplest and most often discussed, transmodulation is proposed to result from ligand-induced receptor heterodimerization (Alroy and Yarden, 1997; Burden and Yarden, 1997; Riese and Stern, 1998). According to this mechanism, a ligand stimulates two receptors to come together. If the two receptors are identical, this is homodimerization; if not, it is heterodimerization. Either way, the two receptors in the dimer become activated by transphosphorylation, and transmembrane signaling is achieved. Several studies argue that erbB receptor extracellular domains are sufficient for their hetero-oligomerization (Qian *et al.*, 1994), and combinatorial receptor (homo- or hetero-) dimerization could be driven by simultaneous binding of bivalent erbB ligands to the extracellular domains of two receptor molecules (Lemmon *et al.*, 1997; Tzahar *et al.*, 1997). Different bivalent ligands could stabilize distinct receptor homo- and/or heterodimers depending on the combination of binding sites that they contain.

An alternative view is that growth factors such as EGF induce only homodimerization of the erbB receptors to which they bind directly. The resulting receptor homodimers may then activate *in trans* the erbB receptors to which the ligand does not bind, through quite different mechanisms. For example, transmodulation of erbB2 by EGF could simply involve phosphorylation of erbB2 as a substrate for the activated EGF receptor. Another possibility (Huang *et al.*, 1998) is that EGF-induced erbB1 homodimers could provide an interface at which dimerization of erbB2 is promoted. ErbB2 could thus become activated by 'proxy' in the context of an (erbB1)₂(erbB2)₂ heterotetramer. A model of this sort could explain the surprising observation that a kinase-negative form of erbB1 can transmodulate erbB2 upon EGF binding (Wright *et al.*, 1995).

In order to determine whether erbB receptor homo- and hetero-oligomerization occur through similar mechanisms, we have studied the effects of ligand binding on the

assembly of isolated erbB receptor extracellular domains. We reported previously that the isolated erbB1 extracellular domain (s-erbB1) homodimerizes quantitatively upon binding to EGF or TGF- α (Lemmon *et al.*, 1997). Here, we show that NRG1- β 1 can also induce homo-oligomerization of the erbB4 extracellular domain. In contrast, ligand-induced hetero-oligomerization appears to be the exception rather than the rule for erbB receptor extracellular domains. While NRG1- β 1 can induce the formation of hetero-oligomers that contain the erbB2 and erbB4 extracellular domains, no evidence could be obtained for EGF-induced formation of any extracellular domain hetero-oligomer. These findings indicate that erbB receptors form homo- and hetero-oligomers through quite different mechanisms, and that transmodulation of erbB receptors is most probably nucleated by a ligand-induced erbB1 or erbB4 homodimer.

Results

High-affinity ligand binding by recombinant s-erbB proteins

To investigate the ligand binding and dimerization properties of soluble erbB receptor extracellular domains (s-erbBs), we first established methods for their production in milligram quantities by secretion from baculovirus-infected Sf9 cells (Figure 1). Using surface plasmon resonance (BIAcore), we next measured binding of each purified s-erbB protein to both EGF and NRG1- β 1 that were immobilized on BIAcore CM-5 sensor chips. The s-erbB proteins were passed across these surfaces at a variety of concentrations, and the maximum response observed was plotted against s-erbB concentration to generate the binding curves shown in Figure 2A. As anticipated, s-erbB1 bound strongly to the EGF-derivatized sensor surface ($K_D = 118$ nM), but not to surfaces carrying NRG1- β 1 or to surfaces with no ligand. Both s-erbB3 and s-erbB4 bound strongly to the NRG1- β 1 surface (K_D values of 249 and 179 nM, respectively; see Table I), but not to the EGF-derivatized surface. In contrast, s-erbB2 did not bind to any of the surfaces tested (Figure 2A). We repeated these experiments using 1:1 mixtures of different s-erbB proteins (e.g. s-erbB2 plus s-erbB3 or s-erbB4) to determine whether free s-erbB proteins might hetero-oligomerize, leading to significant alterations in their apparent ligand-binding affinities. In these studies, mixing s-erbB proteins had no detectable influence on their ligand-binding properties (not shown), arguing that s-erbB hetero-oligomers (if they form) do not bind the immobilized ligands with a significantly higher affinity than single s-erbB species.

s-erbB1 and s-erbB4 homo-oligomerize upon ligand binding, while s-erbB3 does not

To analyze ligand-induced dimerization of s-erbB proteins, we employed multi-angle laser light scattering (MALLS) and sedimentation equilibrium analytical ultracentrifugation, both of which give information on molecular mass changes that is independent of molecular shape (Cantor and Schimmel, 1980).

Multi-angle laser light-scattering studies. MALLS allows the weight-averaged molecular mass (\bar{M}_w) of proteins in solution to be measured rapidly over a wide

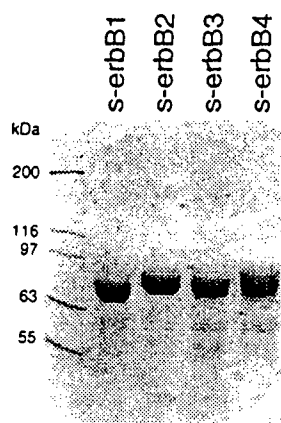


Fig. 1. SDS-PAGE (7.5%) of the purified s-erbB proteins used for analysis of ligand-induced homo- and hetero-oligomerization. Purified protein (15 μ l) was loaded at a concentration of 1 mg/ml, and the gel was stained with Coomassie blue. Molecular mass standards were loaded in the left-most lane, and are marked.

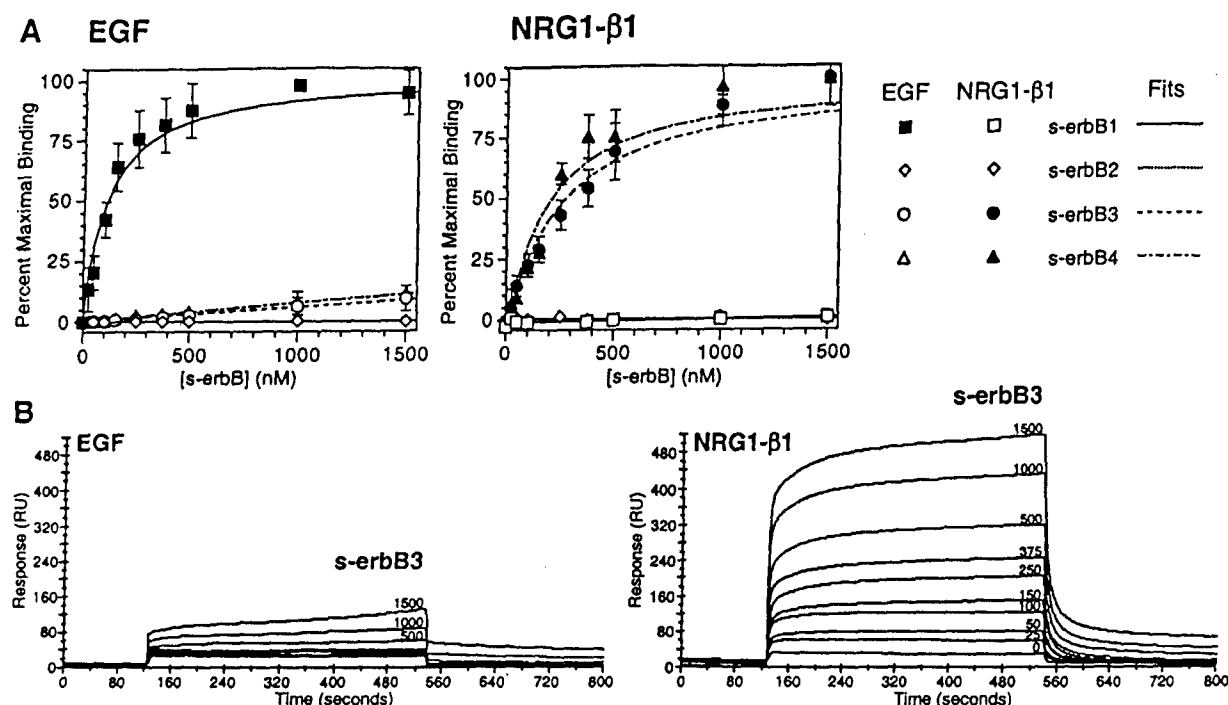


Fig. 2. (A) Data for binding of s-erbB1, s-erbB2, s-erbB3 and s-erbB4 to EGF (left) and NRG1-β1 (right), immobilized on a BIAcore sensor chip. Best fits to the data, assuming a simple association model, are shown. Errors are standard deviations from the mean of at least four independent determinations at each point. K_D values represented by the best fits are listed in Table I. (B) Representative raw BIAcore data for s-erbB3 flowed in parallel over a biosensor chip derivatized with EGF (left) and NRG1-β1 (right) at a series of different concentrations (marked on each curve in nM).

Table I. Ligand binding by s-erbB proteins

Ligand	K_D (nM)			
	s-erbB1	s-erbB2	s-erbB3	s-erbB4
EGF	118 ± 41	none	>10 ⁴	>10 ⁴
NRG-β1	>10 ⁵	none	249 ± 80	179 ± 10

K_D values measured using BIAcore for binding of s-erbB proteins to immobilized EGF and NRG1-β1. Means of at least four independent determinations are quoted alongside their standard deviations.

range of protein concentrations (see Materials and methods). MALLS measurements gave an \bar{M}_w value of 77 ± 8 kDa for purified s-erbB1 alone. When EGF is titrated effectively into an s-erbB1 solution (with fixed s-erbB1 concentration), \bar{M}_w increases in a linear fashion until one molar equivalent of EGF has been added to s-erbB1 (Figure 3A). At this point, \bar{M}_w is 2.2-fold higher than that measured for s-erbB1 alone, suggesting EGF-induced formation of a dimeric complex containing two EGF molecules plus two molecules of s-erbB1, as we have observed with other methods (Lemmon *et al.*, 1997). No further increase in \bar{M}_w is seen when EGF is added in excess, arguing that higher order oligomers of s-erbB1 do not form. The curve through the data in Figure 3A represents the results expected if EGF binds to monomeric s-erbB1 with a $K_D = 118$ nM (Table I), and the resulting 1:1 (EGF:s-erbB1) complex dimerizes completely. The K_D for this dimerization event (which is complete at 4 μ M s-erbB1) appears to be <0.1 μ M, based on additional

MALLS studies at low concentration and gel filtration experiments (not shown).

Similar MALLS studies of s-erbB4 gave a \bar{M}_w of 82 ± 6 kDa that increased by a factor of >2 as NRG1-β1 was added (Figure 3B). In this case, the maximum \bar{M}_w value was not reached until more than two equivalents of NRG1-β1 had been added. Furthermore, the final \bar{M}_w value (~235 kDa) was higher than expected for a dimeric s-erbB4-NRG1-β1 complex. These data therefore suggest that NRG1-β1 is able to induce formation of s-erbB4 oligomers that are larger than dimers. Without more detailed analysis at significantly higher protein concentrations and at larger excesses of ligand, we cannot determine the maximum oligomeric state. However, an increase of nearly 3-fold in \bar{M}_w (at an NRG1-β1:s-erbB4 ratio of 3:1) is equally consistent with the formation of s-erbB4 trimers and with the formation of a mixture that contains 50% of the s-erbB4 as dimers plus 50% as tetramers.

We also used MALLS to analyze the ability of NRG1-β1 to induce s-erbB3 oligomerization. As shown by a single data point in Figure 3B (and confirmed in centrifugation studies described below), addition of a 2-fold excess of NRG1-β1 did not increase the \bar{M}_w measured for s-erbB3 above that measured for s-erbB3 alone (90 ± 4 kDa). This finding is consistent with a previous report (Horan *et al.*, 1995), and does not reflect a lack of NRG1-β1 binding by s-erbB3 (see Figure 2B and Table I). Addition of neither EGF (Figure 3A) nor NRG1-β1 (not shown) altered the value measured for s-erbB2 (78 ± 10 kDa), as expected since neither ligand binds to this protein (Figure 2A).

Analytical ultracentrifugation. Sedimentation equilibrium experiments gave the same results for ligand-induced

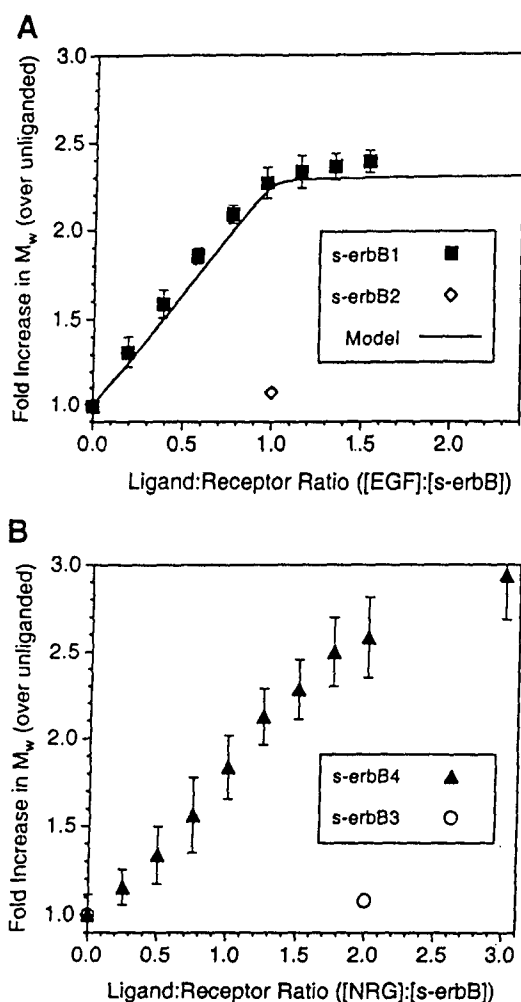


Fig. 3. MALLS studies of EGF-induced homodimerization of s-erbB1 (A) and NRG1- β 1-induced homo-oligomerization of s-erbB4 (B). The weight-averaged molecular mass (M_w) of s-erbB1:EGF mixtures (relative to s-erbB1 alone), as determined by MALLS (see Materials and methods), is plotted against the EGF:s-erbB1 ratio in the mixture. Quantitative EGF-induced s-erbB1 homodimerization is shown (filled squares). The solid line represents the expected results for a model in which EGF binds s-erbB1 with a K_D of 118 nM, and the resulting 1:1 complex dimerizes with a K_D of 100 nM (see text). The single open diamond in (A) shows one point for a similar experiment with s-erbB2, demonstrating that s-erbB2 does not dimerize when EGF is added (see also Figure 4B). In (B), the same experiment is shown for NRG1- β 1 binding to s-erbB4 (filled triangles), which it causes to oligomerize. Also in (B), a single point (open circle) shows the failure of NRG1- β 1 to induce s-erbB3 homo-oligomerization. Error bars correspond to the standard deviations for the mean of three or more experiments. The concentration of s-erbB protein was 4 μ M in each experiment.

s-erbB protein homo-oligomerization. Figure 4 shows typical data from sedimentation equilibrium experiments (at 6000 r.p.m.) in which 5 μ M samples of each s-erbB protein were centrifuged both with (filled symbols) and without (open symbols) a 2-fold molar excess of the most relevant growth factor ligand. Data obtained with the ligand-free s-erbB proteins can be fit, using a model that assumes a single non-ideal species, to give molecular mass estimates of 81 ± 1 kDa (s-erbB1), 80 ± 3 kDa (s-erbB2), 82 ± 7 kDa (s-erbB3) and 81 ± 3 kDa (s-erbB4). The residuals for these fits, plotted above the data in Figure 4,

are both small and random, indicating good fits. When EGF is added to s-erbB1 (Figure 4A), or NRG1- β 1 is added to s-erbB4 (Figure 4D), the radial distribution plots suggest a substantial increase in molecular mass (with material accumulating at higher radii). Since the molecular masses of EGF and NRG1- β 1 are only 6 and 8 kDa, respectively (Lemmon *et al.*, 1997; data not shown), and those of s-erbB1 and s-erbB4 are \sim 80 kDa, this effect can only be explained by homo-oligomerization of the s-erbB proteins upon addition of the relevant growth factor. The data for s-erbB:ligand mixtures can be fit using a model that assumes two ideal species: the ligand-receptor complex and excess ligand. Using this model, the masses of s-erbB1-EGF and s-erbB4-NRG1- β 1 complexes are estimated as 159 ± 10 kDa and 146 ± 18 kDa, respectively (residuals for these fits are shown in Figure 4A and D), consistent with the ligand-induced oligomerization of these extracellular domains seen by MALLS. In other sedimentation experiments (not shown), TGF- α and HB-EGF were also found to induce formation of s-erbB1 homo-oligomers (assumed dimers). As with MALLS, sedimentation equilibrium studies of s-erbB4:NRG1- β 1 mixtures at higher s-erbB4 concentrations and larger ligand excesses (not shown) suggested that NRG1- β 1 induces formation of s-erbB4 oligomers larger than dimers. However, we have not yet been able to determine whether these are trimers or mixtures of different oligomers.

In contrast to the findings for s-erbB1 and s-erbB4, no indication of ligand-induced oligomerization was seen when EGF was added to s-erbB2 (Figure 4B), or when NRG1- β 1 was added to either s-erbB3 (Figure 4C) or s-erbB2 (see below). The data for the s-erbB2:EGF mixture were best fit as a combination of free EGF and free s-erbB2 (82 ± 12 kDa), and those for the s-erbB3:NRG1- β 1 mixture fit best as free NRG1- β 1 (8 kDa) plus a 1:1 s-erbB3-NRG1- β 1 complex of 83 ± 17 kDa.

ErbB1 and erbB2 extracellular domains do not heterodimerize upon EGF binding

Having confirmed that EGF induces s-erbB1 homodimerization, and that NRG1- β 1 induces s-erbB4 homo-oligomerization, we next investigated the ability of erbB ligands to induce heterodimerization of erbB receptor extracellular domains. As described in the Introduction, the most well-studied example of erbB receptor transmodulation involves erbB1 and erbB2. Since EGF induces complete homodimerization of s-erbB1, we expected from the simple heterodimerization model for erbB receptor transmodulation that EGF should also induce the formation of s-erbB1-s-erbB2 heterodimers.

Contrary to these expectations, heterodimer formation could not be observed in MALLS studies when EGF was added to a 1:1 mixture of s-erbB1 and s-erbB2. Instead, EGF induced homodimerization of s-erbB1 in the mixture, while s-erbB2 remained monomeric. As shown in Figure 5A, titration of EGF into a solution containing 4 μ M (crossed-squares) or 8 μ M (filled squares) s-erbB1 alone caused complete dimerization. M_w reached a maximum value (\sim 2-fold) after addition of EGF to \sim 4 and 8 μ M, respectively, as expected for the formation of a 2:2 EGF:s-erbB1 dimer. If EGF-induced heterodimeriza-

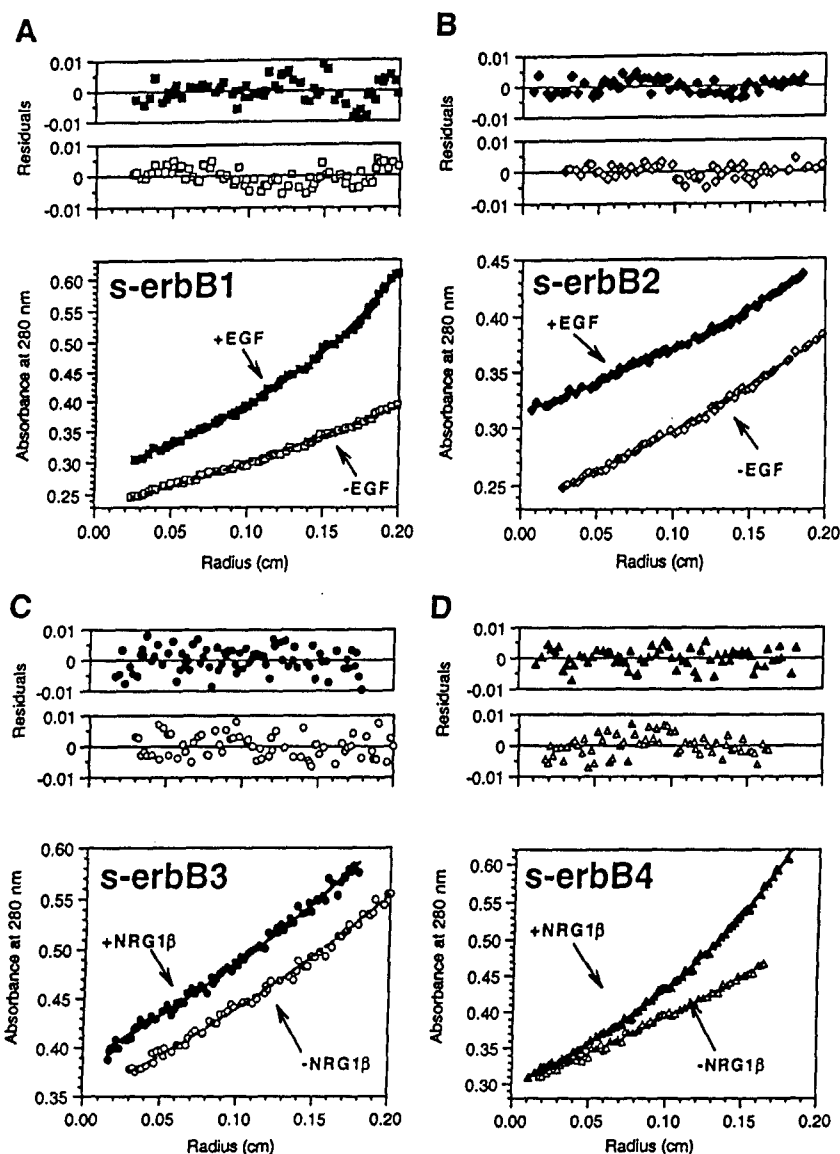


Fig. 4. Representative sedimentation equilibrium analytical ultracentrifugation data for analysis of s-erbB homo-oligomerization induced by EGF or NRG1- β 1. In each case, open symbols represent s-erbB protein without added ligand, which is fit as a single non-ideal species. Filled symbols represent samples to which a 2-fold molar excess of the noted ligand has been added. As discussed in the text, fits to these data are with two ideal species (complex plus excess free ligand)—fixing the mass of the ligand and floating the mass of the complex. Purified s-erbB protein was used at 5 μ M for each sample. All experiments shown were performed at 6000 r.p.m. Repeats at 9000 and 12 000 r.p.m. gave the same results. Residuals for the fits described above are shown, and are seen to be both small and random, indicative of a good fit. EGF induced homo-oligomerization of s-erbB1 only, while NRG1- β 1 induced homo-oligomerization of s-erbB4 only.

tion of s-erbB1 with s-erbB2 were similarly strong, MALLS data for a 1:1 s-erbB1:s-erbB2 mixture (8 μ M total receptor) should resemble that seen for 8 μ M s-erbB1 alone. However, EGF addition to such a 1:1 mixture (diamonds in Figure 5A) induced a maximum \bar{M}_w increase of only 1.6-fold, and this maximum was reached at 4 μ M, not 8 μ M, total EGF. Homodimerization of just s-erbB1 (at 4 μ M) in this mixture would be maximal at 4 μ M EGF according to the data in Figure 3A. Furthermore, a 1.6-fold increase in \bar{M}_w is exactly what is expected if s-erbB1 homodimerizes (yielding 174 kDa s-erbB1 dimers at 2 μ M) while s-erbB2 remains monomeric (80 kDa s-erbB2 monomers at 4 μ M). Therefore, EGF does not induce heterodimerization of s-erbB1 with s-erbB2—or at least the K_D for this heterodimerization event is sufficiently

weak to be undetectable under these conditions (where s-erbB1 homodimerization is complete).

Sedimentation equilibrium experiments also argue strongly against EGF-induced s-erbB1-s-erbB2 heterodimerization. For a set of experiments performed at 6000 r.p.m., the natural logarithm of absorbance at 290 nm (proportional to protein concentration) is plotted in Figure 5B against $(r^2 - r_0^2)/2$, where r is the radial position in the sample, and r_0 the radial position of the meniscus. For an ideal single species, this plot is linear and the gradient of the line $[\bar{M}\omega^2(1 - \bar{V}_2\rho)/RT]$ is proportional to the molecular mass (M) of the ideal species (Cantor and Schimmel, 1980). The data for s-erbB1 or s-erbB2 alone fit well to a straight line with a gradient that suggests a molecular mass of ~80 kDa in each case. When two molar

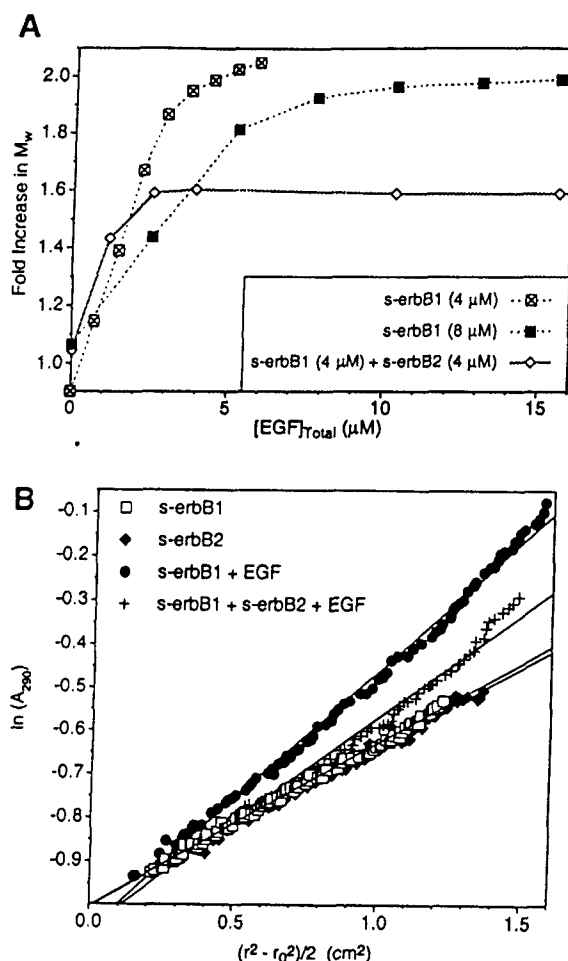


Fig. 5. (A) A MALLS experiment demonstrating that, while EGF induces complete homo-dimerization of s-erbB1 at 4 μM (crossed squares) or 8 μM (filled squares), it does not induce the formation of heterodimers between s-erbB2 and s-erbB1 (open diamonds). The experiment was performed as described for Figure 3. With 4 μM s-erbB1, complete dimerization is seen after addition of 4 μM EGF (note that the horizontal axis here is EGF concentration, and not ligand:receptor ratio). With 8 μM s-erbB1, addition of 8 μM EGF is required for complete dimerization. When the 1:1 s-erbB1:s-erbB2 mixture is studied, with a total s-erbB protein concentration of 8 μM , only 4 μM EGF is required for maximal dimerization, and the maximum fold increase in M_w is consistent only with a mixture of s-erbB1 homodimers and s-erbB2 monomers. Lines are drawn to guide the eye, and do not represent fits to the data. (B) Plots of the natural logarithm of absorbance at 290 nm (monitoring protein concentration) against a function of the radius squared ($r^2 - r_0^2$)/2 (see text for explanation) for sedimentation equilibrium analytical ultracentrifugation data obtained at 6000 r.p.m. with s-erbB1 and s-erbB2. For an ideal single species, this representation of the data should appear as a straight line with a gradient proportional to the molecular mass (see text). When analyzed alone, both s-erbB1 (open squares) and s-erbB2 (filled diamonds) yield good straight lines, with gradients proportional to their monomeric molecular masses (see also fits in Figure 4). Each sample contained a total s-erbB concentration of 10 μM . The increase in gradient for the s-erbB1/s-erbB2/EGF mixture (crosses) is consistent with the formation of s-erbB1 homodimers only.

equivalents of EGF were added to s-erbB1, the gradient of the best straight line (filled squares) was increased substantially over that for s-erbB1 alone, because of EGF-induced s-erbB1 homodimerization. When the same

excess of EGF was added to a 1:1 s-erbB1:s-erbB2 mixture (two EGFs added per s-erbB molecule), the data fit less well to a straight line (indicating multiple species), and the gradient of the best line was increased only slightly over that for s-erbB1 or s-erbB2 alone. Similar experiments at substantially higher receptor concentrations also failed to provide evidence for erbB1-erbB2 hetero-oligomerization. Thus, as seen with MALLS, analytical ultracentrifugation studies suggest that EGF induces homodimerization of s-erbB1 in a s-erbB1:s-erbB2 mixture, while s-erbB2 remains monomeric.

These biophysical studies show that the isolated extracellular domains of erbB1 and erbB2 do not associate with one another in a heterodimer (or any other oligomer) upon EGF addition, whereas s-erbB1 homodimerizes efficiently upon EGF binding (Figures 3, 4 and 5) and EGF-dependent co-immunoprecipitation of intact erbB1 and erbB2 has been reported by many groups. In studies not shown, we attempted to detect s-erbB1-s-erbB2 interactions using chemical cross-linking and co-immunoprecipitation approaches, and obtained only negative results (although s-erbB1 homodimers could be seen readily by chemical cross-linking). We therefore suggest that, while the extracellular domain is sufficient for EGF-induced homodimerization of erbB1, extracellular domains are not capable of driving receptor hetero-oligomerization. Before concluding this, however, an important caveat must be considered. Since erbB2 has no known ligand, we cannot validate the functional integrity of Sf9 cell-derived s-erbB2 by virtue of its ligand binding, as was possible with s-erbB1, s-erbB3 and s-erbB4 (Figure 2). However, we believe that s-erbB2 is functional, since it appears to form NRG1-induced hetero-oligomers with s-erbB4 (see below).

ErbB1 and erbB4 extracellular domains do not hetero-oligomerize upon EGF or NRG1- β 1 binding

Evidence for hetero-oligomerization (or transmodulation) of erbB1 and erbB4 upon treatment of cells with either EGF or NRG has been reported by several groups (Riese *et al.*, 1995, 1996; Cohen *et al.*, 1996; Zhang *et al.*, 1996; F.E.Jones *et al.*, 1999). We therefore used analytical ultracentrifugation to investigate whether EGF and NRG1- β 1 induce s-erbB1-s-erbB4 heterodimerization. Since we know that s-erbB1 and s-erbB4 are both competent to homo-oligomerize upon binding of EGF and NRG1- β 1, respectively, we can be confident that these proteins are functionally active.

A series of sedimentation equilibrium experiments was performed with 1:1 mixtures of s-erbB1 and s-erbB4, with the same total receptor concentration (8 μM) in each case (Figure 6). When no ligand was added, the gradient of the straight line through the data gives an average monomeric molecular mass of ~80 kDa. Addition of EGF to a concentration twice that of total receptor (i.e. two EGF molecules per s-erbB1 molecule plus two EGF molecules per one s-erbB4) increases the gradient of the straight line only slightly (circles in Figure 6), suggesting that some oligomerization is induced. Addition of only NRG1- β 1 to the same final concentration gives a similar result (triangles in Figure 6). Since ligand is not limiting in either of these cases, we hypothesized that these small increases in gradient result from homo-oligomerization of

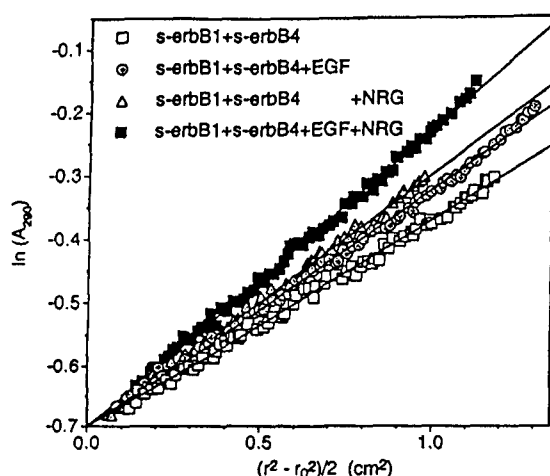


Fig. 6. Analytical ultracentrifugation data, presented as $\ln(\text{Abs})$ against $(r^2 - r_0^2)/2$ plots, to study s-erbB1-s-erbB4 hetero-oligomerization. The s-erbB1:s-erbB4 mixture (8 μM total [s-erbB]) without ligand gives a straight line with the gradient expected for monomeric protein (open squares). Addition of EGF alone (16 μM) or NRG alone (16 μM) results in a modest increase in molecular mass that is consistent with homo-oligomerization of one species only (gray circles and triangles, respectively). Addition of both EGF and NRG (8 μM each) results in a substantially larger increase in the gradient (black squares), indicating that both species homo-oligomerize independently, and do not form hetero-oligomers (see text for explanation).

just s-erbB1 when EGF is added, and of just s-erbB4 when NRG1- β 1 is added. If this is true, an identical sample containing the same total ligand concentration, but as a 1:1 mixture of EGF and NRG1- β 1 (i.e. with two EGF molecules per s-erbB1 molecule plus two NRG1- β 1 molecules per one s-erbB4), should give a substantially steeper gradient, by inducing independent homo-oligomerization of both s-erbB1 and s-erbB4. Indeed, the steepest line in Figure 6 (filled squares) shows this to be the case, arguing that s-erbB1 and s-erbB4 do not form hetero-oligomers under these conditions—with either EGF or NRG1- β 1.

Evidence for NRG1- β 1-induced hetero-oligomerization of s-erbB4 and s-erbB2

The experiments described above show that EGF does not induce hetero-oligomerization of s-erbB1 with s-erbB2 or s-erbB4. Other experiments showed that EGF does not induce the formation of s-erbB1-s-erbB3 or s-erbB2-s-erbB3 hetero-oligomers, and that NRG1- β 1 does not drive the interaction of s-erbB1 with s-erbB3 (not shown). Therefore, although EGF-induced s-erbB1 homodimerization is highly efficient, s-erbB1 does not participate in formation of any s-erbB hetero-oligomer. Furthermore, EGF cannot induce the formation of any s-erbB hetero-oligomer. To compare these properties of EGF with those of NRG1- β 1, we next tested the ability of NRG1- β 1 to induce formation of a series of s-erbB dimers (Figure 7). Using linearized sedimentation equilibrium data as a qualitative guide, Figure 7A, B and C shows that NRG1- β 1 induces homo-oligomerization of s-erbB4 (see also Figures 3B and 4D), but not s-erbB2 or s-erbB3. The data for s-erbB4 homo-oligomerization (from Figure 7A) are superimposed upon all other graphs in Figure 7 to aid

comparison. NRG1- β 1 addition to a s-erbB2:s-erbB3 mixture caused a slight increase in the gradient of the best straight line through the data (Figure 7D), suggesting that there may be very weak hetero-oligomerization of these proteins (although much weaker than s-erbB4 homo-oligomerization). The data obtained with a s-erbB3:s-erbB4 mixture (Figure 7E) are most consistent with NRG1- β 1 inducing independent homo-oligomerization of s-erbB4, with no effect on s-erbB3 (as seen for NRG1- β 1 addition to a s-erbB1/s-erbB4 mixture)—and therefore do not suggest a hetero-oligomerization event.

Figure 7F shows the most interesting of these results, and represents the only data in this study that argue for ligand-induced s-erbB hetero-oligomerization. In the absence of NRG1- β 1, sedimentation of the s-erbB2:s-erbB4 mixture is indistinguishable from that of unliganded s-erbB4. When NRG1- β 1 is added, sedimentation of the s-erbB2:s-erbB4 mixture is almost identical to that seen with s-erbB4 alone (at the same total s-erbB concentration). This argues that NRG1- β 1 addition induces the same increase in average molecular mass regardless of whether all of the s-erbB molecules in the sample are s-erbB4, or half of them are s-erbB2. There are two possible explanations for this. One is that NRG1- β 1 can induce homo-oligomerization of s-erbB2 (as well as that of s-erbB4), which Figure 7B shows to be false. The other explanation is that hetero-oligomers containing s-erbB2 plus s-erbB4 are induced by NRG1- β 1 with an efficiency similar to s-erbB4 homo-oligomerization. Independent MALLS studies (not shown) also showed that the addition of 1.5-fold molar excess of NRG1- β 1 induces the same increase in weight-averaged molecular mass for a 1:1 s-erbB2:s-erbB4 mixture as it does for a solution of s-erbB4 alone, again suggesting NRG1- β 1-induced s-erbB2-s-erbB4 hetero-oligomerization.

Discussion

Using analytical ultracentrifugation and MALLS, we have shown that EGF induces efficient homodimerization of the EGF receptor extracellular domain (s-erbB1), but does not induce formation of any detectable hetero-oligomers (or other homo-oligomers) of erbB receptor extracellular domains. Similar studies with NRG1- β 1 showed that this ligand induces efficient homo-oligomerization of the erbB4 extracellular domain (s-erbB4), but no other s-erbB homo-oligomers. The s-erbB4 oligomers induced by NRG1- β 1 appear to be larger than dimers, although we have not yet established their maximum size. As well as inducing s-erbB4 homo-oligomerization, NRG1- β 1 appears to stabilize the formation of hetero-oligomers containing both s-erbB4 and s-erbB2. The qualitative results of our studies are summarized in Table II.

Comparisons with previous studies

The K_D value reported in Table I for EGF binding by s-erbB1 (118 nM) is comparable with values previously reported (100–500 nM) for EGF binding by monomeric s-erbB1 (Greenfield *et al.*, 1989; Günther *et al.*, 1990; Hurwitz *et al.*, 1991; Lax *et al.*, 1991; Zhou *et al.*, 1993; Brown *et al.*, 1994; Lemmon *et al.*, 1997). However, the data in Figure 3A suggest that the s-erbB1 used here dimerizes at least 15-fold more strongly upon EGF binding

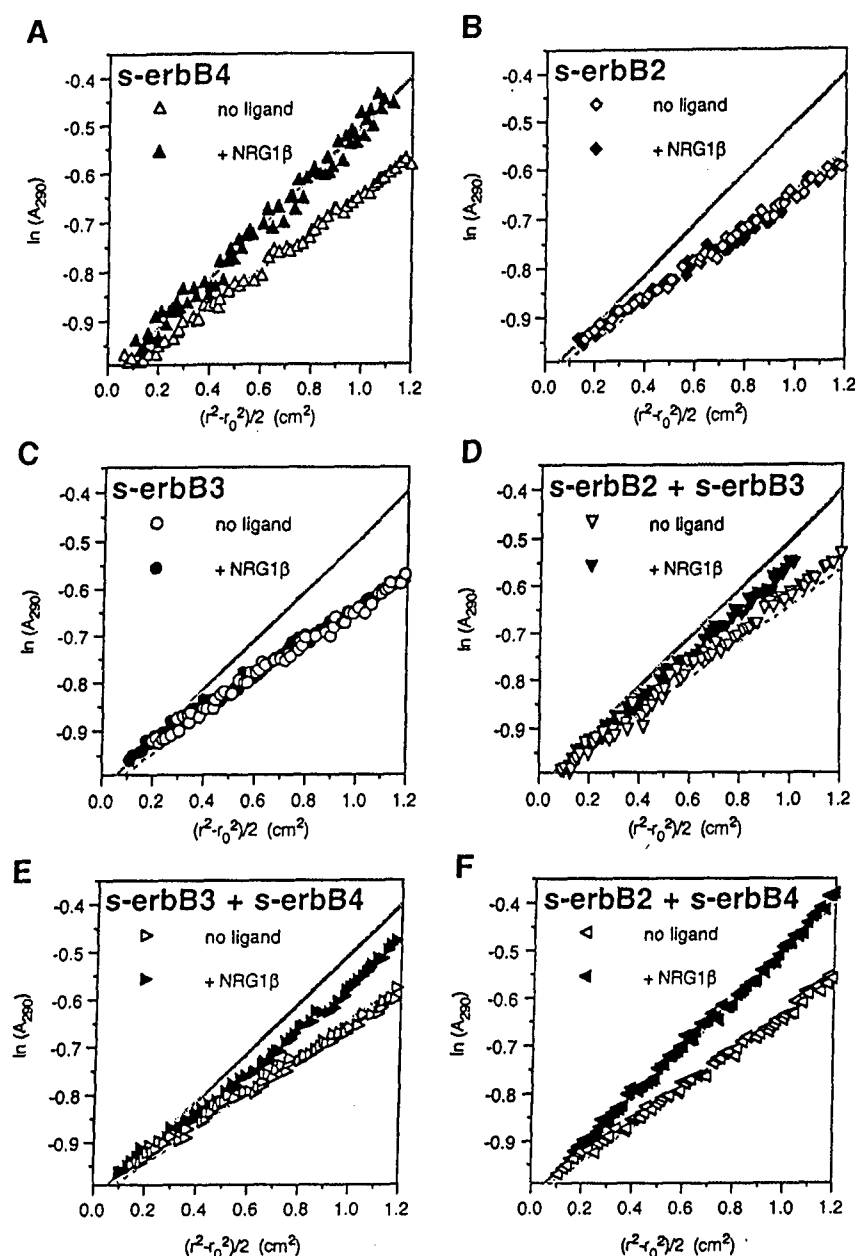


Fig. 7. Plots of $\ln(\text{Abs})$ against $(r^2 - r_0^2)/2$ for different pairwise mixtures of s-erbB2, s-erbB3 and s-erbB4 with (open symbols) and without (filled symbols) added NRG1- β 1. (A) The increase in gradient of the $\ln(\text{Abs})$ against $(r^2 - r_0^2)/2$ plot that results from NRG1- β 1-induced homo-oligomerization of s-erbB4. Lines corresponding to these data are superimposed in gray on each other graph in the figure. (B and C) NRG1- β 1 fails to induce homo-oligomerization of s-erbB2 or s-erbB3. The data in (D) suggest that s-erbB2 and s-erbB3 may form very weak hetero-oligomers upon NRG1- β 1 addition. As seen for s-erbB1 and s-erbB2 in Figure 5B, the data in (E) argue that s-erbB3 does not form hetero-oligomers with s-erbB4. The correspondence (F) of the line for the s-erbB2/s-erbB4 + NRG1- β 1 sample with that for NRG1- β 1-induced s-erbB4 oligomers shown in (A) indicates that NRG1- β 1 can induce formation of s-erbB2-s-erbB4 hetero-oligomers (see text for details). Experiments were performed with a total s-erbB concentration of 10 μM , to which was added a 2-fold molar excess of NRG1- β 1.

than material used in our earlier studies. Whereas the K_D for dimerization of a 1:1 EGF:s-erbB1 complex was estimated previously as 3.3 μM (Lemmon *et al.*, 1997), in which case it would be <50% dimeric in Figure 3A, the protein used in this study remained completely dimeric at concentrations as low as 250 nM (not shown). This difference may reflect the fact that, rather than using chaotropes to elute the protein from immunoaffinity columns, s-erbB1 produced for this study was purified under milder conditions, using metal affinity chromatography (see Materials and methods).

The K_D value reported for s-erbB3 binding to the EGF domain of NRG1- β 1 (249 nM; Table I) is ~10-fold weaker than the value reported for its binding to full-length NRG1- β 2 in analytical ultracentrifugation studies (Horan *et al.*, 1995). This difference may reflect the use of alternative NRG1- β isoforms in the two studies or, more likely, a contribution to s-erbB3 binding by regions of full-length NRG1- β 2 outside the EGF domain (although the EGF domain is sufficient for all known biological activities of NRG1; Holmes *et al.*, 1992). In agreement with our findings (Figures 4C and 7), Horan *et al.* (1995)

Table II. Summary of ligand-induced s-erbB oligomers observed

	s-erbB1		s-erbB2		s-erbB3		s-erbB4	
	EGF	NRG1- β 1	EGF	NRG1- β 1	EGF	NRG1- β 1	EGF	NRG1- β 1
s-erbB1	homo	—	—	—	—	—	—	—
s-erbB2	—	—	—	—	—	hetero (weak)	—	hetero
s-erbB3	—	—	—	—	—	—	—	—
s-erbB4	—	—	—	—	—	—	—	homo

did not detect s-erbB3 homodimerization or s-erbB2-s-erbB3 heterodimerization upon NRG1- β 2 binding.

Implications for erbB receptor oligomerization

As stated in the Introduction, we set out to test the hypothesis that the mechanism of erbB receptor transmodulation involves simple formation of receptor heterodimers upon binding to one or another bivalent ligand (Alroy and Yarden, 1997; Lemmon *et al.*, 1997; Tzahar *et al.*, 1997). We found that, in common with almost every other RTK extracellular domain that has been studied (Lemmon and Schlessinger, 1994; Heldin, 1995), the erbB1 and erbB4 extracellular domains form homo-oligomers upon binding to their respective ligands (EGF and NRG1- β 1). As with other well-characterized examples, this homo-oligomerization may be driven by bivalent erbB ligand binding. However, we could only detect the formation of one of the six possible pairwise s-erbB hetero-oligomers; s-erbB4 forming co-oligomers with s-erbB2 upon NRG1- β 1 binding. EGF did not induce any s-erbB oligomer other than s-erbB1 homodimers, and our data suggest that the one hetero-oligomer that we could detect (s-erbB2-s-erbB4) is likely to be larger than a dimer.

These observations suggest that the simple erbB receptor heterodimerization hypothesis, in which ligand binding drives the heteromeric association of two different erbB receptors through their extracellular ligand-binding domains, is false. Instead, our findings argue that the mechanisms of ligand-induced erbB receptor homo- and hetero-oligomerization must be fundamentally different. In particular, the fact that ligand-induced erbB1 and erbB4 homo-oligomerization can be recapitulated with the isolated extracellular domains of these receptors, while hetero-oligomerization cannot, suggests that regions outside the extracellular domain are required for heteromeric, but not homomeric, interactions of the intact forms of these receptors.

A model for 'homodimer-nucleated' erbB receptor transmodulation

There are $\sim 10^4$ – 10^5 erbB1 or erbB4 receptors on the surface of a typical EGF- or NRG-responsive cell. For a cell with a radius of 8 μ m, this receptor density translates to an effective local concentration of 0.1–3 μ M at the very least. More reasonable estimates that account for orientation effects would be 10–100 times higher (Grasberger *et al.*, 1986). All experiments presented herein were performed with s-erbB proteins at concentrations of 4–10 μ M, mimicking the effective erbB receptor concentra-

tion at the cell surface. Since liganded s-erbB1 and s-erbB4 homo-oligomerize so strongly under these conditions, we suggest that homo-oligomerization of the intact membrane-anchored receptors is likely to be the first response to ligand binding *in vivo*. It seems unlikely that ligand-induced hetero-oligomerization events that we cannot detect in the studies described here (driven by regions outside the extracellular domains) would compete with these strong, directly ligand-induced, homomeric interactions. We therefore suggest that the ligand-induced erbB receptor hetero-oligomers seen in many studies of intact erbB receptors are 'nucleated' by ligand-induced erbB1 or erbB4 homo-oligomers, and most probably represent something larger than a heterodimer. Huang *et al.* (1998) have suggested a similar model, as outlined in the Introduction, in which a ligand-induced homodimer of one receptor (e.g. erbB1) transactivates a second receptor (e.g. erbB2) by inducing its dimerization. In the resulting heterotetramer, the two molecules of the second (unliganded) receptor could activate one another through trans-autophosphorylation, and may be identical or different (if different, the 'secondary dimerization' observations made by Gamett *et al.*, 1997 could be explained). A 'homodimer-nucleated' hetero-tetramer model of this sort could explain the initially surprising finding that a kinase-negative mutant of erbB1 is nonetheless able to mediate EGF-induced transmodulation of erbB2 (Wright *et al.*, 1995). According to the model, an EGF-induced homodimer of the erbB1 mutant would transactivate erbB2 by inducing erbB2 homodimerization (and consequent activation) within the context of a heterotetramer—the kinase activity of erbB1 would not be required. The model could also explain how an erbB2 mutant with its intracellular domain deleted can inhibit transmodulation of endogenous erbB2 in a dominant-negative manner (Jones and Stern, 1999).

While this homodimer-nucleated heterotetramer model may explain transmodulation mediated by erbB1 or erbB2, it cannot readily explain the formation of erbB2-erbB3 hetero-oligomers. We and others (Horan *et al.*, 1995; Tzahar *et al.*, 1997) have failed to detect NRG-induced homodimerization of the erbB3 extracellular domain using biophysical or cross-linking methods. However, NRG-induced homo-oligomerization of intact (or truncated) erbB3 in cells has been detected in chemical cross-linking studies (Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1997). Unlike erbB1 or erbB4, erbB3 appears to require more than just the extracellular domain for its ligand-induced homo-oligomerization. Tzahar *et al.* (1997) have presented evidence suggesting that transmembrane domain

interactions may be important for both homo- and hetero-oligomeric interactions of erbB3. An NRG-induced erbB3 oligomer, stabilized by such interactions, could transmodulate erbB2 by inducing its 'proxy' dimerization in the model discussed above (see also Huang *et al.*, 1998).

Relationship of hetero-oligomer formation to ligand binding

Despite the fact that it does not bind either ligand independently, overexpression of erbB2 increases the NRG-binding affinity of cells that express erbB3 (Sliwkowski *et al.*, 1994; Karunakaran *et al.*, 1996) and the EGF-binding affinity of cells that express erbB1 (Karunakaran *et al.*, 1996). In an effort to understand these effects, Sliwkowski and colleagues investigated how forced heterodimerization of erbB receptor extracellular domains alters their ligand-binding properties. Hetero- (and homo-) dimerization was forced by fusing erbB receptor extracellular domains to the (dimeric) hinge and F_c portions of IgG₁ heavy chain. Heterodimeric IgG fusions containing the erbB2 extracellular domain alongside that of erbB3 or erbB4 bound NRG1- β significantly more strongly than erbB3 or erbB4 homodimer fusion (Fitzpatrick *et al.*, 1998; J.T.Jones *et al.*, 1999). In contrast, a heterodimer containing the extracellular domains of erbB2 and erbB1 was indistinguishable from the equivalent erbB1 homodimer in its binding to EGF, TGF- α , HB-EGF or betacellulin (J.T.Jones *et al.*, 1999). This difference suggests that erbB2 enhances NRG and EGF binding through distinct mechanisms. While NRG binding may be enhanced simply by receptor extracellular domain heteromerization, some other mechanism must be invoked for the enhancement of cellular EGF binding by overexpression of erbB2 (Karunakaran *et al.*, 1996). Our studies of s-erbB oligomerization suggest a similar distinction: while the isolated extracellular domains cannot recapitulate ligand-induced erbB1-erbB2 hetero-oligomerization, at least NRG-induced erbB2-erbB4 heteromerization could be reproduced with the soluble s-erbB proteins studied here.

Conclusions

Regardless of the precise mechanism of ligand-induced erbB receptor hetero-oligomerization, the results presented here show that isolated extracellular domains reproduce ligand-induced homomeric interactions of erbB receptors more faithfully than their reported heteromeric interactions. This finding alone argues that the mechanisms for homo- and hetero-oligomerization of the erbB receptors must differ. Our data therefore provide strong evidence against the simple heterodimerization hypothesis that we set out to test. Rather, in agreement with suggestions made by other groups (Gamett *et al.*, 1997; Huang *et al.*, 1998), we suggest that the ligand-induced erbB homo-oligomers that can be formed with isolated extracellular domains nucleate larger erbB hetero-oligomers through interactions that may also involve other regions of the receptor. Transphosphorylation within these larger 'homodimer-nucleated' hetero-oligomers may be responsible for erbB receptor transmodulation.

Materials and methods

Generation of s-erbB constructs

A fragment of human erbB1 cDNA directing expression of residues 1–642 (1–618 of the mature sequence), followed by a hexahistidine tag and stop codon, was subcloned into pFastBac1 (Life Technologies Inc). The 1955 bp fragment was generated by PCR, introducing a unique *Bgl*III site immediately before the initiation codon and a unique *Xba*I site that follows the introduced stop codon. The 1955 bp *Bgl*III–*Xba*I-digested PCR product was ligated into *Bam*HI–*Xba*I-digested pFastBac1. To minimize the risk of PCR artifacts, a 1260 bp *Eco*RI–*Apa*I fragment of this PCR-derived clone was swapped for the equivalent region from the original erbB1 cDNA. A fragment of human erbB2 cDNA, directing expression of residues 1–647 (1–628 of the mature sequence), was generated similarly. In this case, a unique *Xba*I site was introduced before the initiation codon, and a unique *Hind*III site was introduced after the histidine tag and stop codon. The 1980 bp *Xba*I–*Hind*III-digested PCR product was ligated into *Xba*I–*Hind*III-digested pFastBac1. An 1880 bp internal fragment of this PCR product, extending from an *Nco*I site at the initiation codon to a unique *Sph*I site, was then swapped for the equivalent fragment from the original erbB2 cDNA.

Fragments encoding human erbB3 residues 1–639 (1–620 of the mature protein) and human erbB4 residues 1–649 (1–624 of the mature protein), with a unique *Bam*HI site at one end and an *Xba*I site at the other, were generated by PCR, and ligated into *Bam*HI–*Xba*I-digested pFastBac1. The sequence of all PCR-derived fragments and their cloning boundaries were confirmed by automated dideoxynucleotide sequencing methods.

Protein production

Typically, 5–10 l of Sf9 cells were grown as a suspension culture in Sf900-II medium (Gibco-BRL) using multiple 1 l spinner flasks that each contained <500 ml of medium (to ensure adequate aeration). At a cell density of 2.5×10^6 cells/ml (viability >98%), freshly amplified high-titer virus stock was added to a multiplicity of infection (m.o.i.) of ~5. Cultures were incubated at 27°C for a further 96 h. Clarified conditioned medium was concentrated 2-fold, and then diafiltered against 3.5 vols of 25 mM Tris-HCl, 150 mM NaCl, pH 8.0 (buffer A), using a Millipore Prep/Scale-TFF 30 kDa cartridge. The solution was concentrated further to ~300 ml prior to loading onto a 5 ml Ni-NTA Superflow column (Qiagen). After extensive washing with buffer A, the column was washed sequentially with two column volumes of buffer A containing 30, 50, 75, 100 and 300 mM imidazole, pH 8.0. Typically, most s-erbB protein eluted in the 75 and 100 mM fractions. Fractions were concentrated in a Centriprep 30 (Amicon), and loaded onto a Pharmacia Superose 6 gel filtration column in 25 mM HEPES, pH 8.0, 100 mM NaCl, from which they eluted as ~85 kDa species. For s-erbB1 and s-erbB4, appropriate gel filtration fractions were pooled, diluted 1.5-fold with 50 mM MES pH 6.0, and were loaded on to an BioScale-S2 cation exchange column (BioRad) pre-equilibrated with 25 mM MES pH 6.0. Protein was eluted with a gradient in NaCl, s-erbB1 eluting at ~200 mM NaCl and s-erbB4 at ~300 mM NaCl. Attempts to purify s-erbB2 and s-erbB3 by ion exchange led to precipitation of the proteins at the low salt concentration required for column binding. Purified s-erbB proteins were buffer exchanged into 25 mM HEPES, 100 mM NaCl, pH 8.0, concentrated to between 20 and 100 μ M, and stored at 4°C. Purity was checked by SDS-PAGE (Figure 1), and concentrations were determined by absorbance at 280 nm using extinction coefficients calculated as described (Mach *et al.*, 1992) of 56 920/M/cm (s-erbB1), 62 460/M/cm (s-erbB2), 63 940/M/cm (s-erbB3) and 74 300/M/cm (s-erbB4). We previously had used quantitative amino acid analysis to measure a value of 58 500/M/cm for s-erbB1 from mammalian cells (Lemmon *et al.*, 1997); this value is within 3% of that calculated according to Mach *et al.* (1992). Calculated extinction coefficients of 18 780/M/cm (EGF) and 5920/M/cm (NRG1- β 1) were also used for determination of ligand concentration.

Approximate final yields of purified protein from 1 l of conditioned medium were 1 (s-erbB1), 0.2 (s-erbB2), 1 (s-erbB3) and 0.5 mg (s-erbB4). Ligands used for this study were purchased from Interger (human EGF) or R & D Systems (human NRG1- β 1).

Multi-angle laser light-scattering (MALLS) studies

A DAWN DSP laser photometer from Wyatt Technologies (Santa Barbara, CA) was used for MALLS studies (Wyatt, 1993). The instrument was used in micro-batch mode, with samples being introduced into the flow cell via a 0.1 μ m filter using a syringe pump. To avoid introduction of air bubbles, concentrated protein solutions were diluted to

working concentrations in degassed buffer, and samples were introduced into the flow cell via a low dead volume multi-port valve that was loaded with several samples and purged of air prior to a series of measurements. Scattering data at all 17 angles were collected until maximum stable scattering for a sample was seen, which can be achieved at flow rates of 2 ml/h with samples of ~300 μ l. Scattering data were collected and analyzed using ASTRA software (Wyatt Technologies) supplied with the instrument. Relative weight-averaged molecular masses were determined from the scattering data collected for a given ligand:receptor mixture (once stabilized) using Debye plots, in which $R(\theta)/K^*c$ is plotted against $\sin^2(\theta/2)$, where θ is the scattering angle; $R(\theta)$ is the excess intensity (I) of scattered light at that angle; c is the concentration of the sample; and K^* is a constant equal to $4\pi^2 n^2 (dn/dc)^2 / \lambda_0^4 N_A$ (where n = solvent refractive index, dn/dc = refractive index increment of scattering sample, λ_0 = wavelength of scattered light and N_A = Avogadro's number). Extrapolation of a Debye plot to zero angle gives an estimate of the weight-averaged molecular mass (\bar{M}_w) (Wyatt, 1993). \bar{M}_w is defined as:

$$\bar{M}_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i}$$

for n moles of i different species with molecular weight M_i .

In ligand titration experiments, the contribution of added ligand to the mass concentration was neglected (see also Lemmon *et al.*, 1997). Since we are interested in dimerization, i.e. only the 'fold increase' in \bar{M}_w , our results are not affected by the value of K^* , of which we are uncertain since we have not determined the extent of glycosylation of the s-erbB proteins accurately. MALLS data are therefore discussed in terms of 'fold increase' in \bar{M}_w over that measured for s-erbB protein alone. Where estimates for \bar{M}_w are reported, mass concentrations were converted from molar concentrations using the molecular weight suggested by the amino acid sequence, and assuming that s-erbB glycoproteins are 20% carbohydrate by mass.

Analytical ultracentrifugation studies

Sedimentation equilibrium experiments employed the XL-A analytical ultracentrifuge (Beckman). Samples were loaded into six-channel epon charcoal-filled centerpieces, using quartz windows. Experiments were performed at 20°C, detecting at 280–300 nm, using three different speeds (6000, 9000 and 12 000 r.p.m.), with very similar results. Solvent density was taken as 1.003 g/ml, and the partial specific volumes of the s-erbB proteins were approximated from their amino acid compositions and the assumption of ~20% carbohydrate as 0.71 ml/g for the purposes described here. Experiments were performed at 5–10 μ M protein. Data were fit using the Optima XL-A data analysis software (Beckman/MicroCal) to models assuming a single non-ideal species for unliganded s-erbB proteins. When ligand was added, a two-species fit was used, in which one of the species was the excess ligand (partial specific volume 0.74 ml/g), which sediments as a 6 kDa (EGF) or 8 kDa (NRG) species (not shown). The molecular mass of the ligand species was fixed in these fits, while the mass and concentration of the receptor species were allowed to float. Goodness of fit was judged by the occurrence of randomly distributed residuals, examples of which are shown in Figure 4. For more complicated mixtures of receptors and ligands, simple qualitative interpretations of analytical ultracentrifugation experiments were made by inspection when possible (see Figures 5B, 6 and 7).

BIAcore studies

BIAcore binding experiments employed a BIAcore 2000 instrument, and were performed in 10 mM HEPES buffer, pH 7.4, that contained 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween-20 at 25°C. The hydrogel matrix of BIAcore CM5 Biosensor chips was activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-[3-(diethylamino)propyl] carbodiimide (EDC). EGF (at 200 μ g/ml) in 10 mM sodium acetate, pH 4.0, or NRG1- β 1 (at 200 μ g/ml) in 10 mM sodium acetate, pH 4.8, was then flowed over the activated surface at 5 μ l/min for 10 min. Non-cross-linked ligand was removed, and unreacted sites were blocked with 1 M ethanolamine, pH 8.5. The signal contributed by immobilized EGF or NRG1- β 1 ranged from 150 to 400 RU, depending on the specific chip. Purified s-erbB proteins at a series of concentrations were each flowed simultaneously over the EGF and NRG1- β 1 (and mock/control) surfaces at 5 μ l/min for 7 min, by which time binding had reached a plateau in each case. The RU value corresponding to this plateau was taken as a measure of s-erbB protein binding, and was corrected for background non-specific

binding and bulk refractive index effects by subtraction of data obtained in parallel using the mock-coupled hydrogel surface. RU values were then converted into percentage maximal binding. This conversion was performed separately for each surface (since levels of immobilization varied); 100% binding was defined for an NRG surface as the highest corrected signal seen with s-erbB3 and s-erbB4 (which were always the same to within 10%), and for an EGF surface the highest corrected signal seen with s-erbB1. Buffer washes between runs were sufficient to bring the RU value back down to baseline. Data were plotted as s-erbB concentration against percentage maximal binding, and fit to a simple binding equation in ORIGIN (MicroCal) to estimate the K_D .

Acknowledgements

We thank Irit Lax, Yossi Schlessinger, Greg Van Duyn and members of the Lemmon laboratory for many valuable discussions and comments on the manuscript. BIAcore experiments were performed in the Biosensor Interaction Analysis Core at Penn. This work was supported by grants from the National Institutes of Health (CA79992 to M.A.L.), the US Army Breast Cancer Research Program (DAMD17-98-1-8232 to M.A.L., and DAMD17-98-1-8228 to K.M.F.), a Scholar Award from the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (to M.A.L.) and the Research Foundation of the University of Pennsylvania.

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Received June 7, 2000; accepted July 11, 2000



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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 May 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-98-1-8232. Request the limited distribution statements for Accession Documents Number ADB258643 and ADB266202 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

A handwritten signature in cursive script, reading "Phyllis M. Rinehart", is positioned above the typed name and title.

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management